

ACTIN, α -ACTININ, AND TROPOMYOSIN INTERACTION IN THE STRUCTURAL ORGANIZATION OF ACTIN FILAMENTS IN NONMUSCLE CELLS

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ABSTRACT

During the spreading of a population of rat embryo cells, approximately 40% of the cells develop a strikingly regular network which precedes the formation of the straight actin filament bundles seen in the fully spread out cells. Immunofluorescence studies with antibodies specific for the skeletal muscle structural proteins actin, α -actinin, and tropomyosin indicate that this network is composed of foci containing actin and α -actinin, connected by tropomyosin-associated actin filaments. Actin filaments, having both tropomyosin and α -actinin associated with them, are also seen to extend from the vertices of this network to the edges of the cell. These results demonstrate a specific interaction of α -actinin and tropomyosin with actin filaments during the assembly and organization of the actin filament bundles of tissue culture cells. The three-dimensional network they form may be regarded as the structural precursor and the vertices of this network as the organization centers of the ultimately formed actin filament bundles of the fully spread out cells.

Actin is a basic structural protein of eucaryotic cells (for review, see Pollard and Weihing, 19). In differentiated skeletal muscle cells, actin filaments interact with myosin filaments and mediate the contraction of these cells by a mechanism which involves the sliding of these two types of filaments past each other (11). In nonmuscle cells, actin filaments have been implicated in a number of cellular functions, such as cytokinesis, endocytosis and exocytosis, cell adhesion to a substratum, cell locomotion, membrane ruffling, and maintenance of cell shape (3, 8, 17, 22, 24). Since myosin is also a component of nonmuscle cells (1, 2), at least some of the functions in which actin filaments are involved may be mediated by a sliding filament mechanism similar to that of skeletal muscle.

Until very recently, the elucidation of the mechanism(s) by which actin filaments mediate specific cellular functions in nonmuscle tissue culture cells has been hampered due to a lack of knowledge both of the cellular structure of these filaments and of the involvement of other structural proteins in the regulation of their assembly and organization. With the demonstration, however, that antibodies to actin can be used in indirect immunofluorescence to localize this protein in both muscle and nonmuscle cells (13, 16), the possibility has opened up that antibodies to other structural proteins from muscle may be used for a similar purpose. Immunofluorescence studies with antibodies to the skeletal muscle structural proteins tropomyosin and α -actinin on nonmuscle cells grown in tis-

sue culture have shown a close association between these two proteins and actin or actin filament bundles in fully spread out cells (12–14, 21).

In order to study the role which these proteins might play in the assembly of actin filament bundles, I have examined by indirect immunofluorescence their localization during the formation of actin filament bundles in the early stages of spreading of rat embryo cells. During the first hours of cell spreading, approximately 40% of the cells develop a very regular polygonal network of actin fibers. The vertices of the network contain α -actinin, but no tropomyosin, whereas the connecting fibers are associated with tropomyosin, but not with α -actinin. Actin fibers containing both α -actinin and tropomyosin are also seen to extend from the vertices of this network to the edges of the cell. The selective staining of this pattern suggests a specific interaction between actin, α -actinin, and tropomyosin during the assembly and organization of actin filament bundles. This transiently formed network may be regarded as the structural precursor, and its vertices may function as organization centers of the ultimately observed actin filament bundles in fully spread out cells.

MATERIALS AND METHODS

Cell Culture

Primary cultures of rat embryo cells were a generous gift of Dr. R. Pollack. The cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and used between the second and third month of subculture for the experiments reported here. Experimental cells were trypsinized at confluence for 5 min with 0.05% trypsin, 0.5 mM ethylenediaminetetraacetate (EDTA) in phosphate-buffered saline (PBS) and seeded on 12-mm round glass coverslips.

Antibody Preparation to Actin and Tropomyosin

Antibodies against actin were prepared in rabbits using as an antigen smooth muscle actin purified to homogeneity after preparative sodium dodecyl sulfate (SDS) polyacrylamide slab gel electrophoresis of purified smooth muscle actomyosin. The purity of this actomyosin preparation is shown in Figure 1 *b*. The details of this antibody preparation as well as the procedure used have been described elsewhere (12, 13). This antibody preparation was found to have properties similar to those obtained with antibodies raised against mouse fibroblast or calf thymus actin (15, 16). It cross-reacted weakly with chicken skeletal muscle actin in double immunodiffusion and specifically stained the actin filaments (I band) of chicken myofibrils in indirect immunofluorescence. As

judged by indirect immunofluorescence, all three antibody preparations give indistinguishable immunofluorescent patterns when tested on tissue culture cells. Furthermore, preabsorption of these antibodies with purified polymeric actin has been shown to render them incapable of staining the filamentous network observed with unabsorbed antisera in tissue culture cells (13). The smooth muscle actin antibodies were used at a final concentration of approximately 1 mg/ml.

Antibodies to tropomyosin were prepared against native chicken skeletal muscle tropomyosin. These antibodies have been found to cross-react in double immunodiffusion with purified smooth muscle (chicken gizzard) and skeletal muscle (chicken or rabbit) tropomyosin (12). The immunofluorescent patterns reported here with this tropomyosin antibody preparation have also been obtained with an antibody preparation to purified chicken skeletal muscle tropomyosin kindly provided by Dr. F. Pepe (University of Pennsylvania, School of Medicine, Philadelphia, Pa.). These antibodies have been previously shown to react specifically with tropomyosin in the I bands of chicken skeletal myofibrils (18). The tropomyosin antibodies were used at a final concentration of 2–2.5 mg/ml.

Antibody Preparation to α -Actinin

Antibodies to α -actinin were prepared against native porcine skeletal muscle α -actinin. The antigen was purified by the method of Goll et al. (10), and was kindly provided by Dr. R. Levine (Medical College of Pennsylvania, Philadelphia, PA.). The purity of the protein that was used as an antigen is shown in Figure 1 *a*. This antigen preparation showed that α -actinin was approximately 90% pure with traces of actin still remaining. However the small amounts of actin present did not alter the specificity of the antiserum because (a) native vertebrate actin (chicken skeletal, smooth muscle, or calf thymus) has been found to be poorly immunogenic (13, 18), (b) neither immunodiffusion nor indirect immunofluorescence on fully spread out tissue culture cells (14) indicated the presence of actin antibodies, (c) preabsorption of the α -actinin antisera with actin free of α -actinin (as judged by analytical SDS-slab gel electrophoresis) did not alter the immunofluorescence patterns reported here, and (d) the antibody stained specifically the Z line of skeletal myofibrils (14); no I band (actin filament) staining was evident even after prolonged incubation of the myofibrils with the α -actinin antibody. The immunization scheme used for preparing antibodies to α -actinin in female white New Zealand rabbits was as follows: days 1 and 8: 0.25 mg administered subcutaneously in Freund's complete adjuvant. Days 15, 17, and 19: 0.1 mg administered intravenously after alum precipitation of the protein (4). Days 22, 24, and 26: 0.2-mg administered intravenously as above. Day 29: 0.3 mg and day 33: 0.5 mg administered intravenously as above. The rabbits were bled by cardiac puncture (30 ml of blood) at day 37, boosted intravenously with 0.2 mg of alum-precipitated

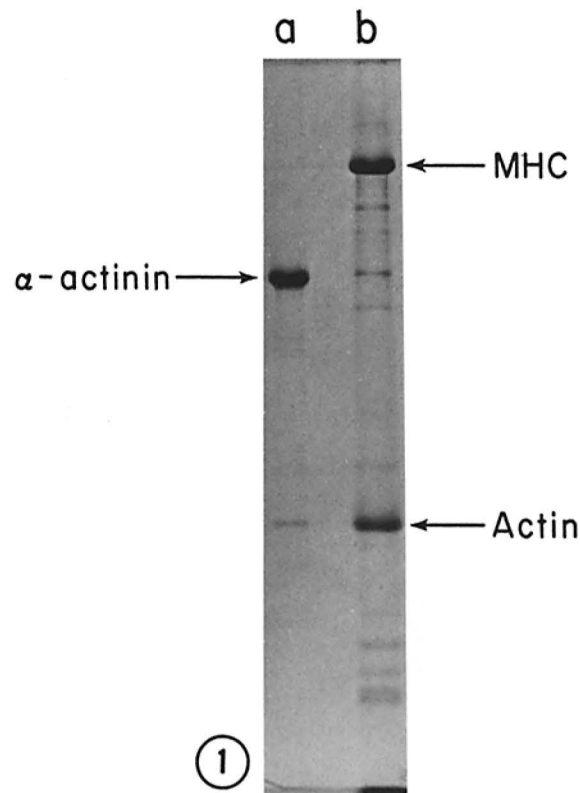


FIGURE 1 Analytical SDS-slab gel electrophoresis on 12.5% polyacrylamide gels of (a) the purified porcine skeletal muscle α -actinin used as an antigen and (b) the partially purified chicken smooth muscle (gizzard) actomyosin used for the preparative purification of actin. *MHC* refers to the myosin heavy chain. Details for the preparation of the gels have been previously described (16).

protein at day 44, and bled subsequently every 5–7 days. They were then boosted every 3 wk. The results reported here have been obtained using the serum from the first bleeding, but identical results have been obtained using the sera from the two subsequent bleedings. The sera were partially purified at 50% ammonium sulfate saturation, and the pellet (globulin) was dialyzed against 0.15 M NaCl, 0.02 M Tris HCl pH 7.8, and stored at -20°C . This antibody preparation gives one precipitin line both by double immunodiffusion against the original antigen (Fig. 2) and by immunoelectrophoresis of the partially purified globulin fraction and subsequent immunodiffusion against the original antigen (lower part of Fig. 3). Immunoelectrophoresis of the antigen and subsequent immunodiffusion against the partially purified globulin fraction revealed a precipitin line with a partial split (upper part of Fig. 3). This “split line” effect is commonly observed in the immunoelectrophoretic analysis of some antigens, most notably in slightly proteolyzed γ -globulin preparations (23). Similarly, in the case of α -actinin, the split line may indicate a slight proteolysis of the antigen; alternatively, it may result from a slight modification of a subpopulation of the α -actinin mole-

cules. However, these two classes of antigens have not been characterized further in this work. Indirect immunofluorescence has further shown that the α -actinin antibody cross-reacts with the Z line of rat myofibrils specifically (14), in accordance with the results reported by Schollmeyer et al. (20). The α -actinin antibodies were used at a final concentration of approximately 0.5 mg/ml.

Indirect Immunofluorescence

The technique of indirect immunofluorescence was a modification of the previously used technique (12), designed to eliminate air-drying of the cells (G. Albrecht-Buehler and E. Lazarides, unpublished observations). Cells grown on 12-mm coverslips were removed from the growth medium, rinsed briefly once in PBS, and fixed for 30 min in 3.5% formaldehyde in PBS at room temperature. The coverslips were then rinsed three times in PBS at room temperature and placed in PBS. They were then successively incubated for 3 min in a 1:1 acetone-water mixture (at 4°C), for 5 min in acetone, for 3 min in a 1:1 acetone-water solution, and finally for 3 min in PBS. The

coverslips were drained on paper, placed on a wet piece of paper, and immediately covered with 20 μ l of the appropriately diluted antisera. Subsequently, they were incubated in a humid atmosphere at 37°C for 90 min. The coverslips were then washed three times in PBS and incubated for 1 h at 37°C with fluorescein-labeled goat antirabbit IgG (Miles Laboratories, Inc., Miles Research Div., Elkhart, Ind.). The goat antirabbit IgG was used at a final concentration of 0.8 mg/ml in PBS and a final absorbance ratio at 280/495 nm of approximately

0.13. At the end of the incubation, the coverslips were washed three times in PBS, once in distilled water, and mounted on a drop of Elvanol (du Pont 51-05, E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). The specimens were viewed under a Zeiss microscope (PMII) equipped with epifluorescence optics. Pictures were taken on Plus X film (Eastman Kodak Co., Rochester, N. Y.) using a 63 \times oil immersion lens. Exposure time was approximately 20–40 s for the actin and tropomyosin antibodies and 45–60 s for the α -actinin antibodies.

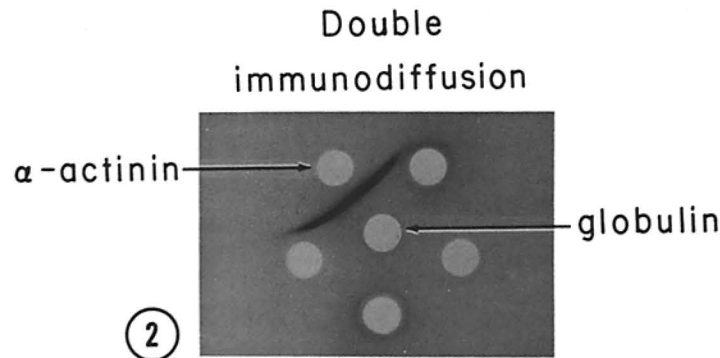


FIGURE 2 Double immunodiffusion of porcine skeletal muscle α -actinin against the partially purified anti- α -actinin globulin preparation. The α -actinin well contained approximately 10 μ g of antigen and the antiglobulin well approximately 400 μ g of protein. Immunodiffusion was carried out for 60 h at 4°C. The plates were subsequently washed for 4 days with 0.15M NaCl, 0.02M Tris HCl pH 7.8, stained with 0.25% Coomassie Brilliant Blue in 50% methanol, 7½% acetic acid, for 30 min and destained with 20% methanol, 7½% acetic acid.

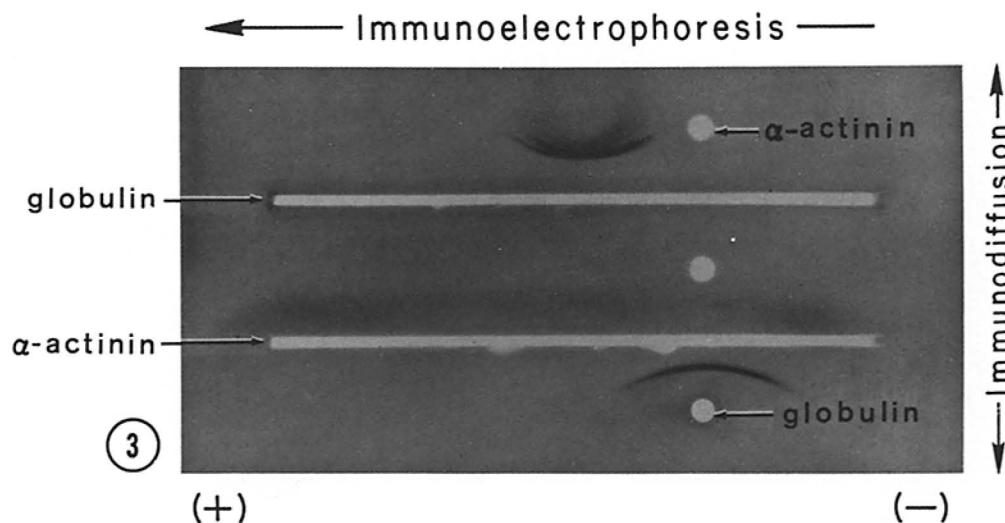


FIGURE 3 Immunoelectrophoresis of the anti- α -actinin globulin fraction (lower part) and of the α -actinin (upper part). Immunoelectrophoresis was carried out in 1% agarose plates buffered with 0.043M sodium barbital, 0.038 M sodium acetate buffer pH 8.8. The running buffer was 0.028 M sodium barbital and 0.025 M sodium acetate buffer pH 8.8. Electrophoresis was done at 150 V for 5 h. Immunodiffusion of α -actinin (lower part) and anti- α -actinin globulin fraction (upper part) as well as washing and staining of the plates was carried out as described in Fig. 2.

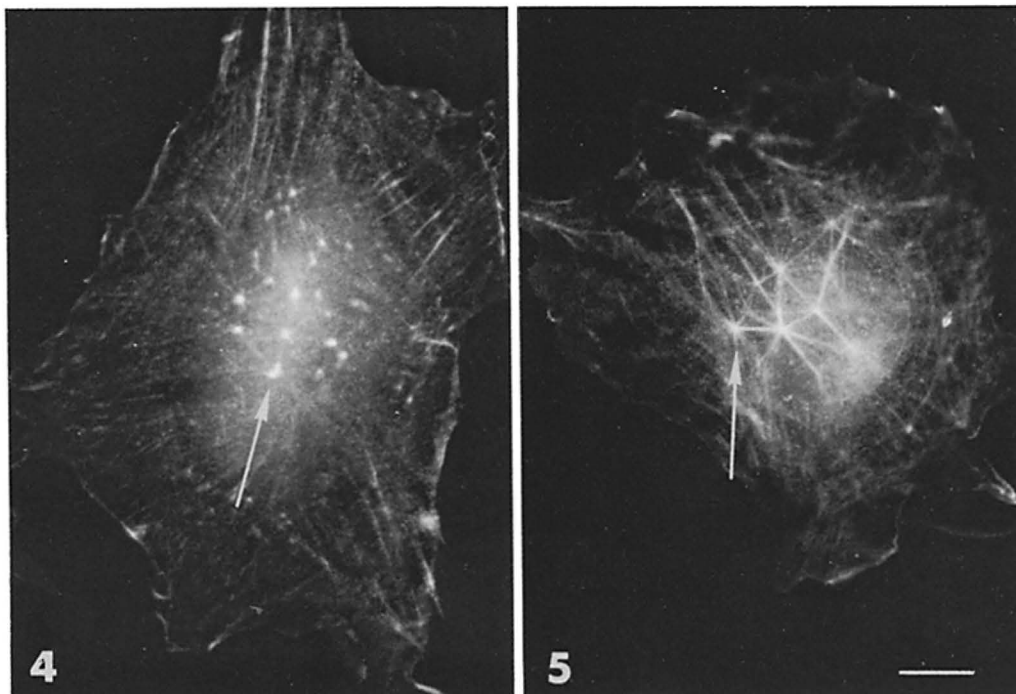
RESULTS

Immunofluorescence Characterization of the Networks

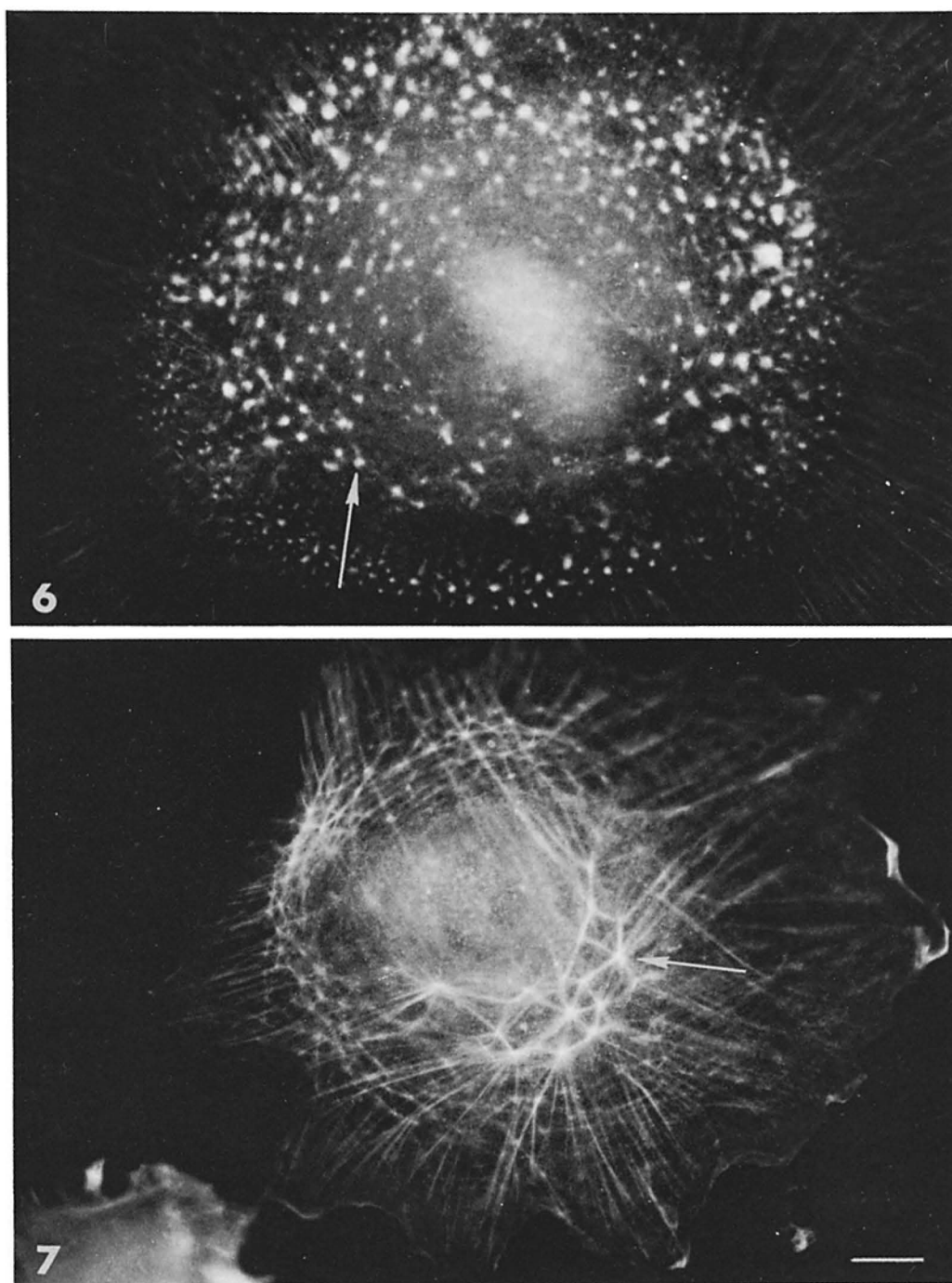
Treatment of cells with trypsin results in their detachment and rounding up, and in a concomitant rapid disassembly of their highly organized actin filament bundles; such cells morphologically resemble a mitotic cell. When replated, they slowly spread out and regain their actin filament bundles, which had previously been lost. These freshly trypsinized, rounded-up cells show a dynamic organization and disorganization of actin filaments in the first 2 h after plating (G. Albrecht-Buehler and E. Lazarides, unpublished observations). During this time, the cells display a diffuse uniform fluorescence when stained with either the actin, the tropomyosin, or the α -actinin antibodies, and the spherical shape of the cells hinders the visualization of any filamentous structures which may have reorganized (13). After 4 h, the cells are sufficiently spread out, and distinct organized patterns of actin-filament bundles become visible by immunofluorescence.

One of the first events that becomes apparent in a large number of cells is the organization of α -actinin into distinct fluorescent foci (Fig. 4). Actin is organized in similar foci, but, in addition, there are actin fiber bundles which connect the foci, producing a strikingly regular network (Fig. 5). The tropomyosin antibody reveals a diffuse fluorescence, and only a minority of the cells show tropomyosin associated with the actin filament bundles (data not shown).

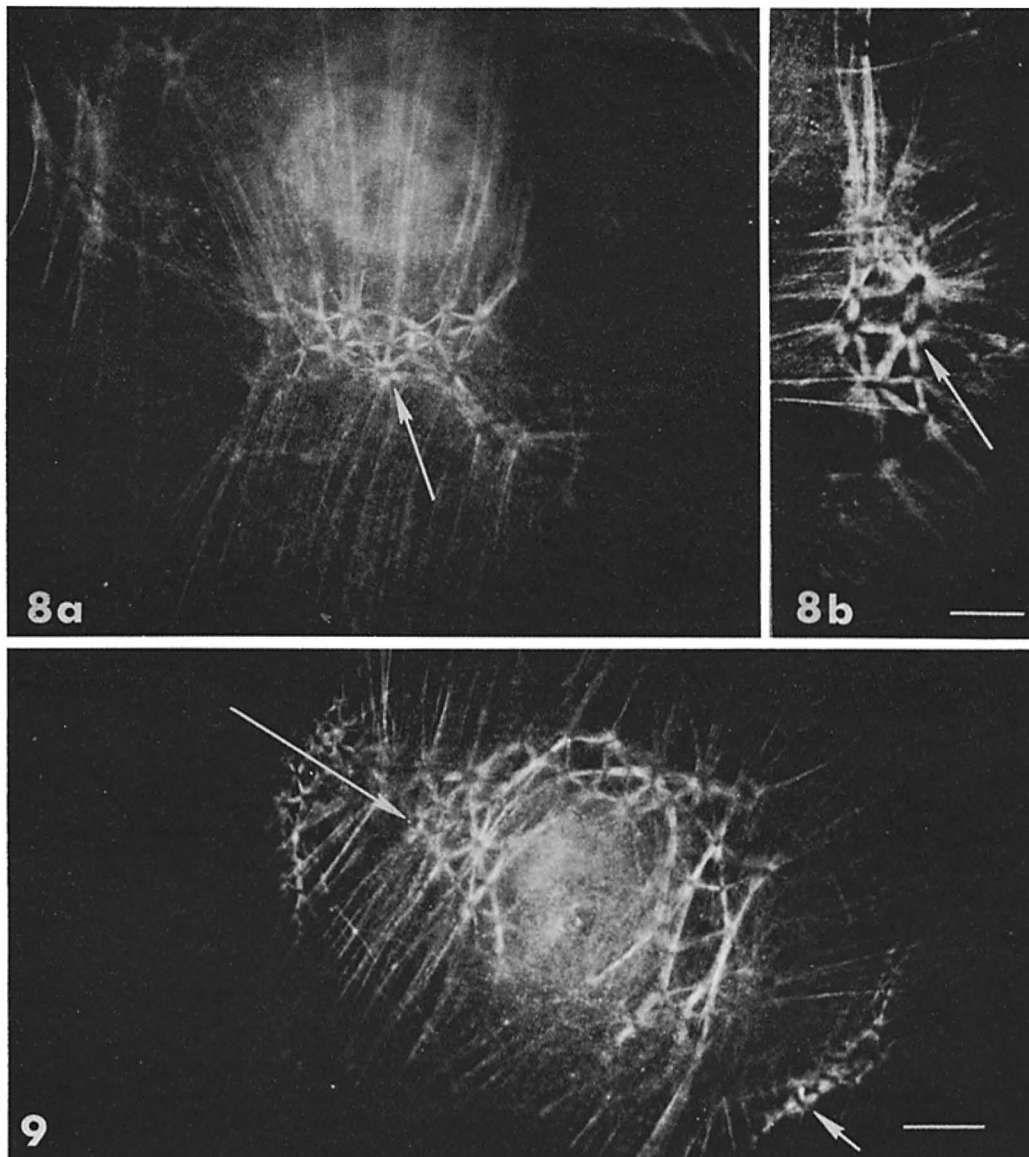
When cells are examined 8 h after plating, the immunofluorescence patterns observed with the actin and α -actinin antibodies are very similar to the earlier patterns, except that each basic pattern is reiterated to cover the whole volume of the spreading cell (Figs. 6 and 7; see also Figs. 10–15 and 18–21). After staining with the tropomyosin antibody, a filamentous network is apparent which is very similar to that seen with the actin antibody. In contrast, however, to both the α -actinin and the actin antibodies, the fluorescence of the tropomyosin antibody is missing at the vertices; its network therefore appears complementary to that observed with the α -actinin antibody (Figs. 8 and 9; see also



FIGURES 4 and 5 Indirect immunofluorescence on a culture of rat embryo cells 4 h after trypsinization and replating with antibodies to α -actinin (Fig. 4) and actin (Fig. 5). The arrows point to the fluorescent foci seen with the α -actinin (Fig. 4) and actin (Fig. 5) antibodies. (bar = 10 μ m).



FIGURES 6 and 7 Indirect immunofluorescence on the same culture of rat embryo cells as that used in Fig. 4 and 5, approximately 7 h after trypsinization and replating with antibodies to α -actinin (Fig. 6) and actin (Fig. 7). The arrows point to the fluorescent foci seen with the α -actinin (Fig. 6) and actin (Fig. 7) antibodies. Note that the actin network in the upper part of the cell in Fig. 7 is out of focus, indicating that the cellular distribution of this network is at a different plane than that which is in focus. (bar = 10 μ m).



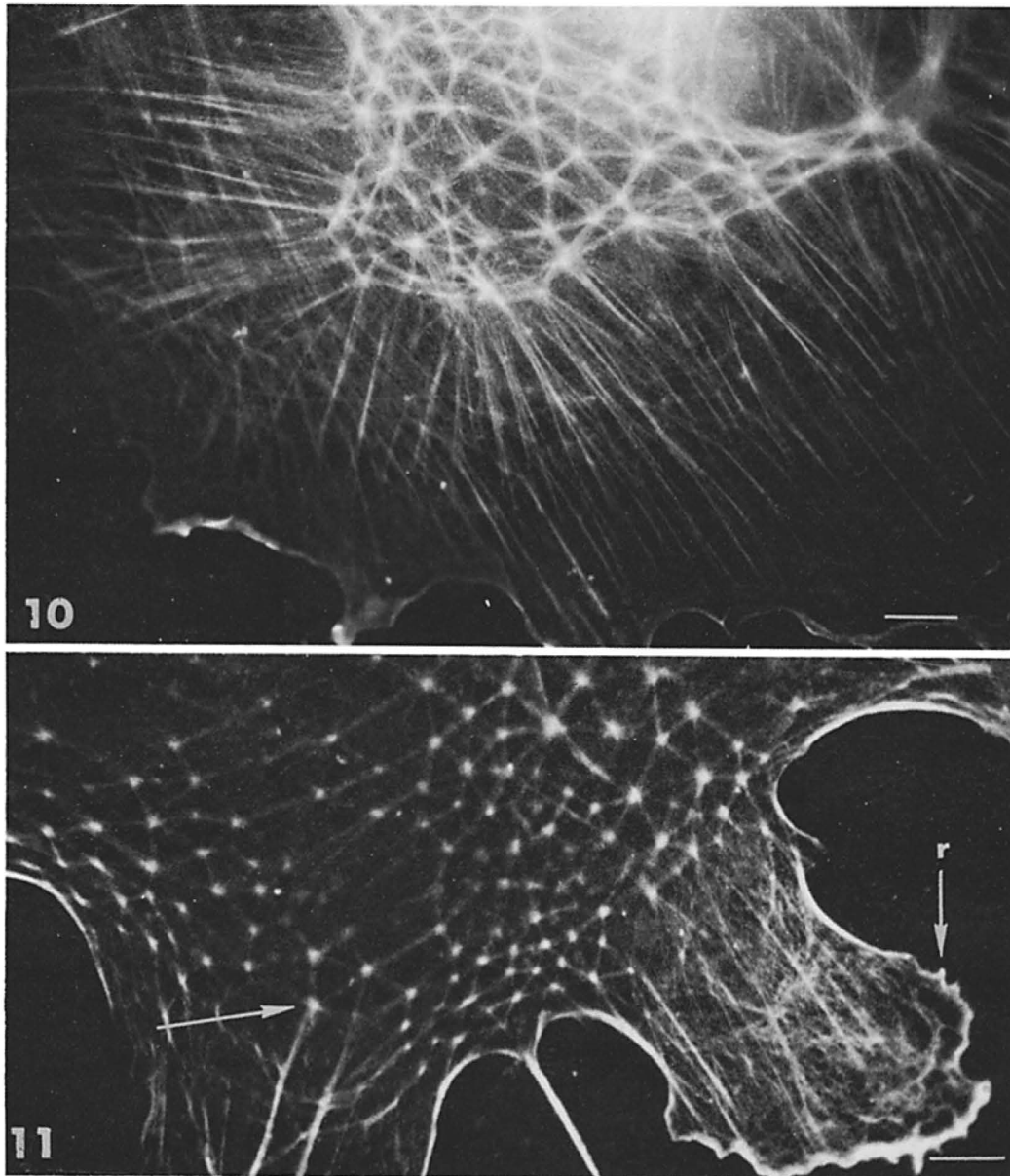
FIGURES 8 and 9 Same cell culture as that used in Figs. 6 and 7, with the difference that indirect immunofluorescence was done with the tropomyosin antibody. The arrows point to the nonfluorescent foci characteristically observed with the tropomyosin antibody. Note the fibers that radiate from the vertices of the network towards the edges of the cell. The bars equal $10\ \mu\text{m}$ for Figs. 8 *a* and 9, and $6\ \mu\text{m}$ for Fig. 8 *b*.

Figs. 16 and 17). The complementarity of the α -actinin and tropomyosin antibodies was confirmed in cells reacted with both antibodies sequentially. Cells treated this way showed both fluorescent foci and fibers connecting these foci, producing a pattern which was indistinguishable from the actin antibody patterns seen in Figs. 5 and 7.

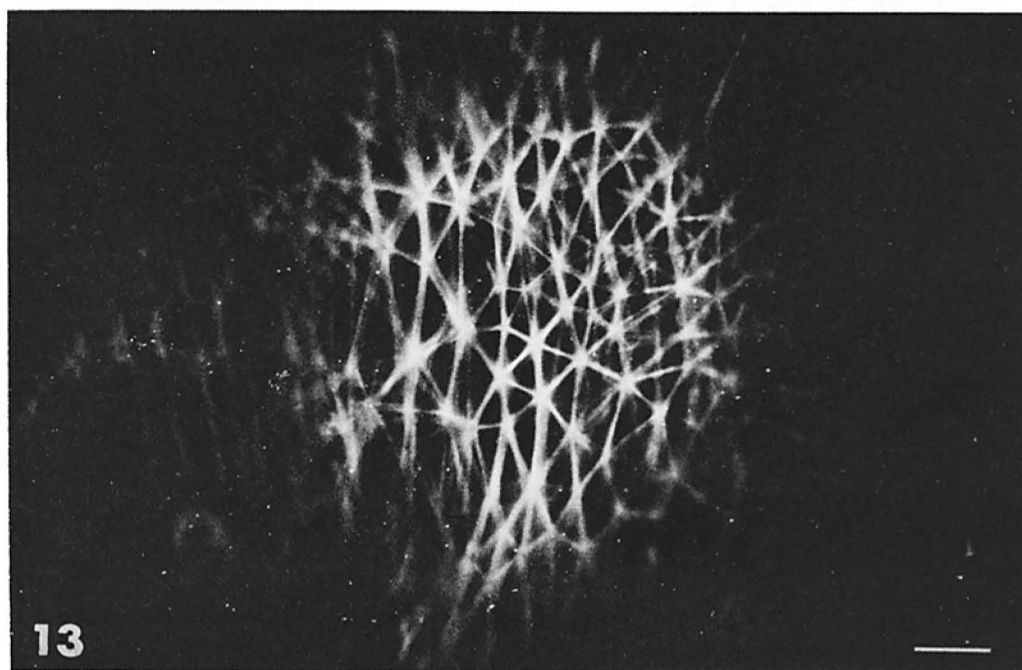
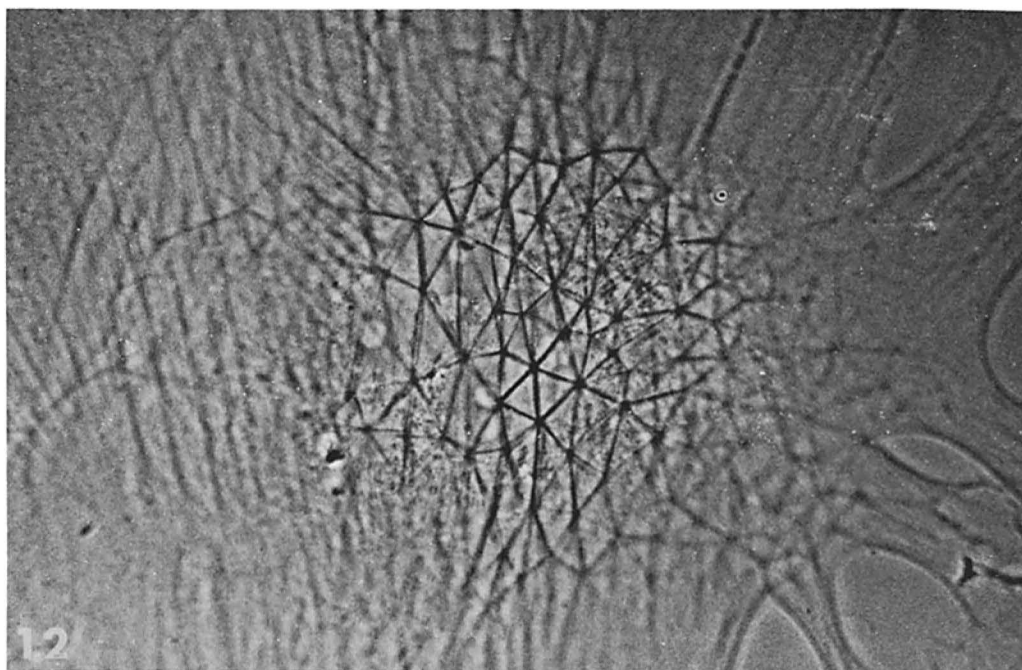
In addition to this network, filament bundles can be found which are connected to the actin- α -actinin foci at only one end and extend all the way to the edge of the spreading membrane (Figs. 10 and 11). Characteristically, however, indirect immunofluorescence indicates that, besides actin, these filament bundles contain both α -actinin and tropomyosin (Figs. 8, 9 and 16–19; see also below).

Yet even at high resolution ($100\times$ oil immersion), these fibers exhibit a continuous fluorescence when stained with the actin antibody. When reacted with the tropomyosin antibody, however, the majority of these fibers show a periodicity of the fluores-

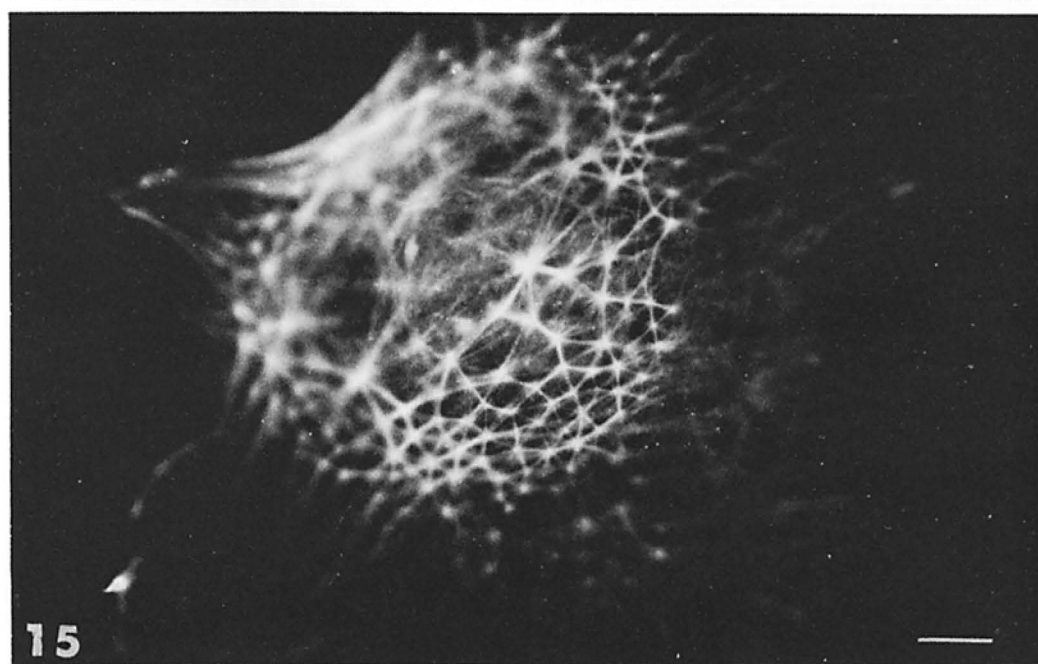
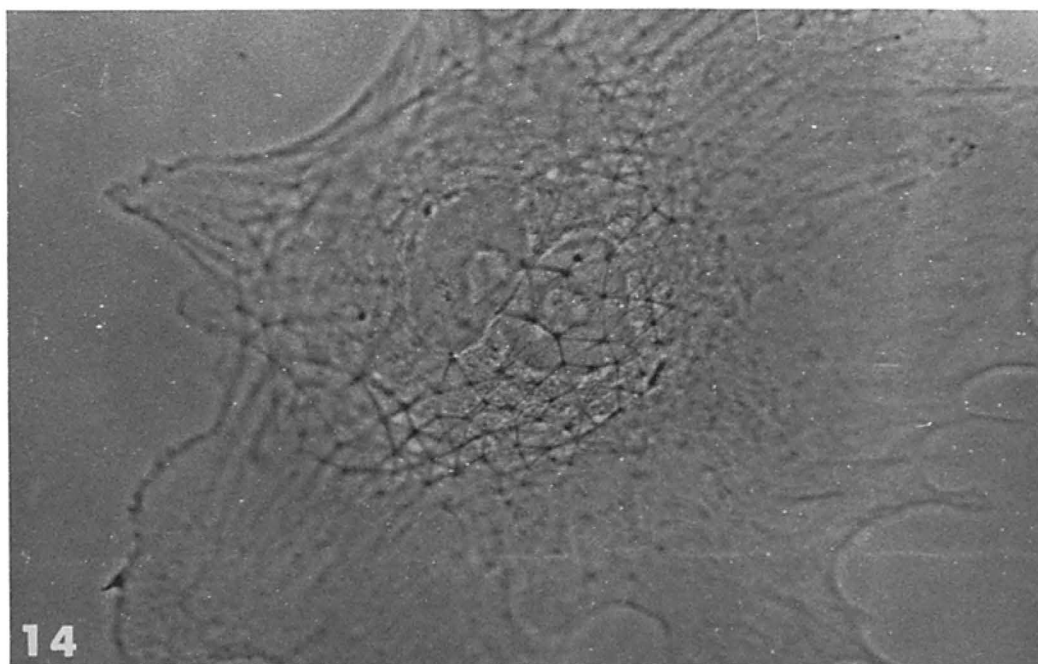
cence ($\sim 1.2\ \mu\text{m}$) which characterizes the distribution of this molecule within the actin filament bundles of fully spread out cells (12). Such a periodicity has not been observed in the fibers that connect the fluorescent foci (Figs. 8, 9, and 17). Similarly,



FIGURES 10 and 11 Same cell culture as that used in Figs. 6 and 7. Indirect immunofluorescence was done on spreading cells $7\frac{1}{2}$ h after plating with the actin antibody. Note the actin fibers that radiate from the vertices of the networks towards the edges of the cell and into areas of membrane ruffling (*r*). The bar in Fig. 10 equals $10\ \mu\text{m}$. The bar in Fig. 11 equals $6\ \mu\text{m}$. Fig. 11 was photographed under oil immersion with a $100\times$ objective.



FIGURES 12-15 Indirect immunofluorescence on a population of rat embryo cells with the actin antibody 8½ h after plating. Cells were reacted with the actin antibody as described in Materials and Methods and photographed with phase contrast optics (Figs. 12 and 14) and epifluorescence optics (Figs. 13 and 15). Note that the networks towards the edge of the cell are out of focus, indicating that they are on a different plane in the cell. (bar = 10 μ m).



with the α -actinin antibody these fibers show a periodicity (Fig. 19) which also characterizes the distribution of this molecule within the actin filament bundles of fully spread out cells, and which is distinct from the periodicity seen with the tropomyosin antibody (13, 14).

Comparison of the Networks with Phase Contrast and Epifluorescence Optics

We have previously shown that the phase contrast fibers observed in fully spread live cells and in cells subjected to the indirect immunofluorescence

technique correspond to the fluorescent fibers seen with the actin antibody (9). Subsequent work showed that this held true for both tropomyosin and α -actinin, indicating that these two molecules were localized within the actin filament bundles (12, 14), although not exclusively there. Figs. 12–15 show that the networks seen with the actin antibody correspond to identical patterns in phase contrast optics in the fixed and antibody-treated cells. Using as a frame of reference the network which is seen in phase contrast optics, it is obvious that the networks that are revealed by the actin and tropomyosin antibodies and the point pattern shown by the α -actinin antibody are in fact representations of the same network. Its vertices coincide with the α -actinin foci (Figs. 18–21), whereas its connecting fibers are the tropomyosin-containing fibers (Figs. 16 and 17). Since the network as a whole is stained by the actin antibody, we can also conclude that the vertices contain both actin and α -actinin and the connecting fibers both actin and tropomyosin. Furthermore, the actin fibers which radiate from the network to the cell edges and which contain both tropomyosin and α -actinin also coincide with their phase contrast fiber counterparts (Figs. 12–21).

Observation of the Networks in Live Cells

These polygonal networks are difficult to observe by phase contrast optics in live cells during this time of spreading. However, some cells show such a pattern unmistakably, especially close to the edge of the cell (Fig. 22). This observation eliminates the possibility that either the network or the fluorescence patterns seen in cells subjected to the indirect immunofluorescence technique and viewed with phase contrast or fluorescence optics are an artifact of the indirect immunofluorescence technique. This point was further substantiated by comparison of the lengths between the vertices of the networks seen in live cells with phase contrast optics and those seen with the actin antibody with epifluorescence optics. Fig. 23 indicates that the lengths correspond very closely, and it further demonstrates the high regularity of the network. In the networks chosen for comparison, the average length between the centers of any two foci was found to be $3.7 \mu\text{m}$ ($\text{SD} = 1.4 \mu\text{m}$) for the actin fluorescence patterns and $3.8 \mu\text{m}$ ($\text{SD} = 1.6 \mu\text{m}$) for the patterns seen in live cells.

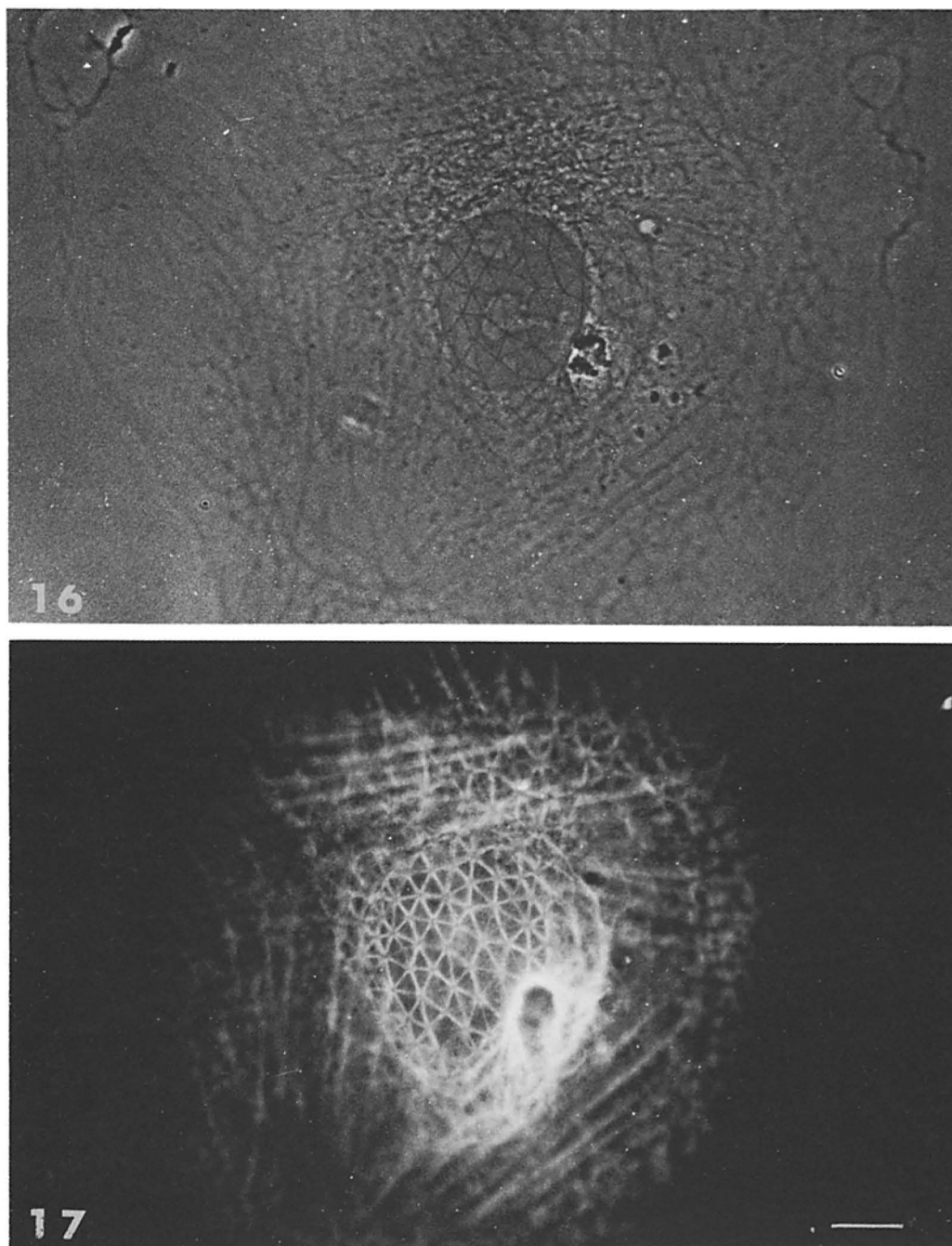
The immunofluorescence patterns described

above with the actin, α -actinin, and tropomyosin antibodies are representative and are commonly observed in populations of spreading rat embryo cells. Similar patterns have been observed in cultures of human skin fibroblasts, in the established mouse cell line BALB/3T3 and in primary rat lung cell cultures. In the rather heterogeneous and asynchronously spreading populations of rat embryo cells, approximately 40% of the cells develop such networks within the first 9 h after plating. Improved synchrony of cell spreading by plating cells in the cold (4°C for 30 min, G. Albrecht-Buehler, personal communication) before incubation at 37°C did not significantly alter the percentage of cells that ultimately develop such patterns, which suggests that the network-forming cells belong to a subpopulation of the corresponding cultures. Detachment of the cells with EDTA alone did not affect the presence of these networks, which indicates that their formation is independent of any trypsin effects on the membrane.

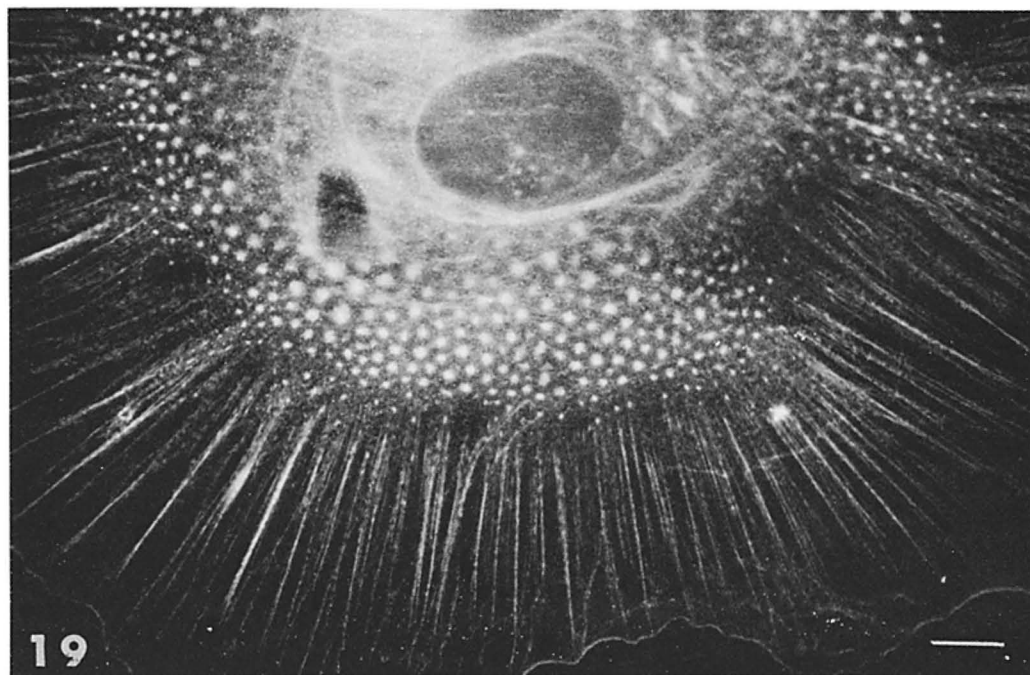
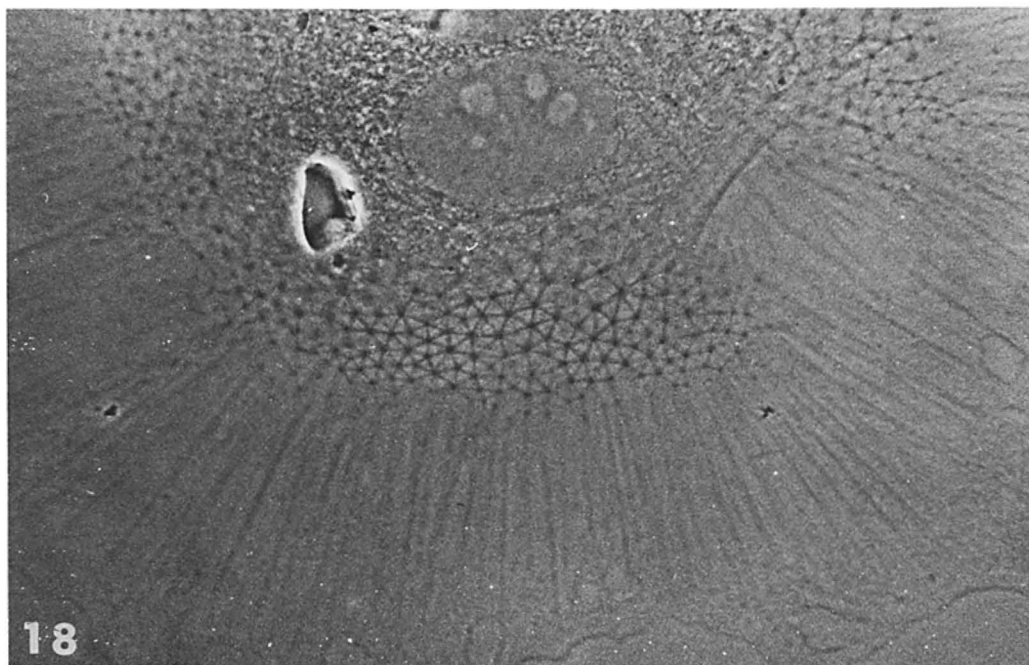
As judged from the immunofluorescence patterns reported above, the fluorescent foci and the fibers are initially clustered preferentially in the nuclear region and, at later stages of spreading, both above (or below) the nucleus and around the nucleus extending all the way to the periphery of the cell. The comparison of the fluorescent and phase contrast fibers in Figs. 12–21 indicates that the networks assume a three-dimensional cellular distribution, thus making it sometimes difficult to focus simultaneously on the whole network.

DISCUSSION

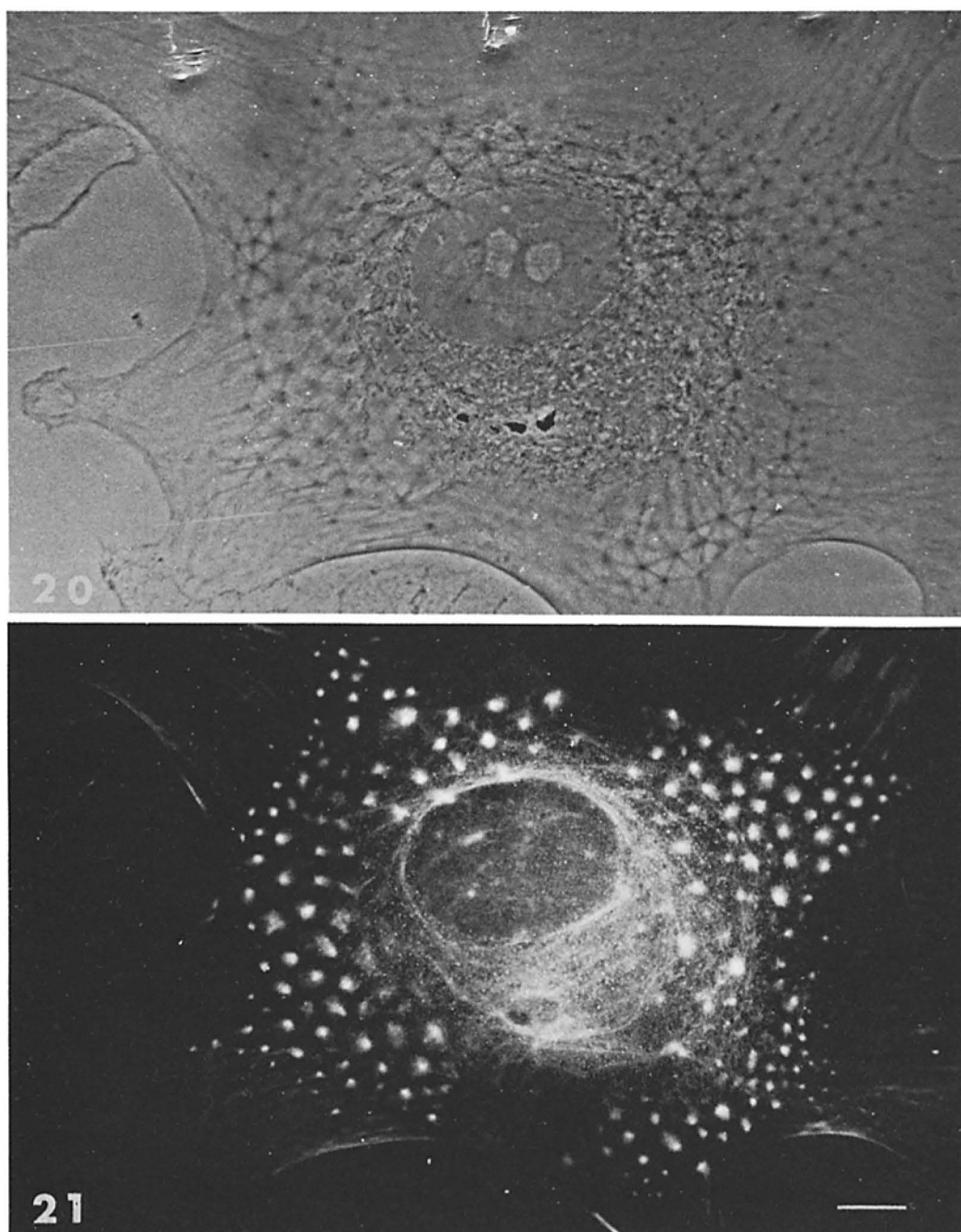
Cells treated with trypsin round up with a simultaneous disaggregation of their highly organized actin fiber bundles that are normally seen in the fully spread out cell. When allowed to spread again, they slowly flatten out with a concomitant reorganization of their lost actin filament bundles. This process of spreading out of a trypsinized cell mimics, morphologically, the spreading of a cell that takes place after mitosis and can be used as a model system in the study of the assembly and organization of actin filament bundles, as well as in investigating the role that other structural proteins might play in such a process. One of the characteristic transient intermediates that is seen to develop during spreading before the formation of the straight actin filament bundles of the fully spread out cells is the highly regular network of actin filaments described above. The immunofluo-



FIGURES 16 and 17 Same as Figs. 12–15 except that the cells were reacted with the tropomyosin antibody. The cells were photographed with phase contrast optics (Fig. 16) and epifluorescence optics (Fig. 17). Note that the vertices of the network in Fig. 17 do not stain. Also note that the networks are focused on the nuclear area. The networks at the edge of the cell are out of focus as described in Figs. 12–15. (bar = 10 μ m).



FIGURES 18-21 Indirect immunofluorescence with the α -actinin antibody on the same population of cells shown in Figs. 12-17 $8\frac{1}{2}$ h after plating. The cells were photographed with phase contrast optics (Figs. 18 and 20) and epifluorescence optics (Figs. 19 and 21). (bar = $10\ \mu\text{m}$).



rescence patterns observed with the actin, α -actinin, and tropomyosin antibodies demonstrate a specific interaction of these three proteins within this polygonal network. One type of interaction is characterized by the formation of foci that contain actin and α -actinin, and the other by the formation

of filament bundles that connect these foci that contain tropomyosin and actin. Although the exact temporal sequence of the appearance of the actin and α -actinin foci is not yet clear, these filamentous complexes may act initially as the nucleation and later as the organization centers for the

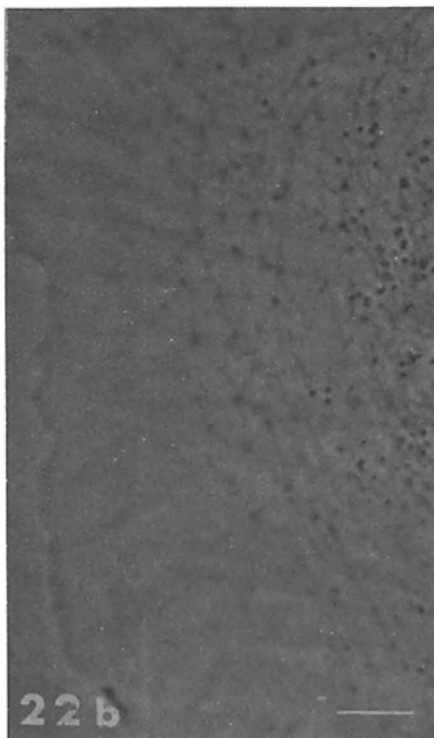
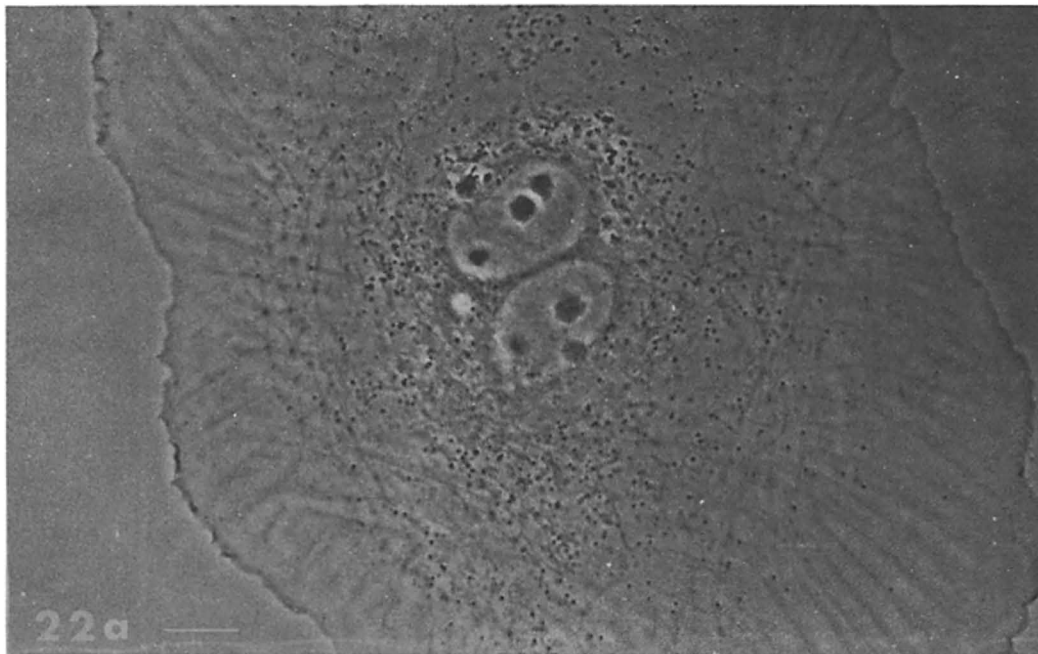


FIGURE 22 Visualization of the network in live cells. Cells were kept live at 37°C in a wax-sealed coverslip approximately 8½ h after plating and photographed with phase contrast optics. In Fig 22 *a* the bar equals 10 μm. Figure 22 *b* is a closer view of a section of the network seen in Figure 22 *a*. The bar equals 7 μm. Note that the network contains phase contrast dense vertices, fibers that connect the vertices, and fibers that extend from the vertices to the edge of the cell.

formation of the fiber bundles that attach at only one end to the vertices of the network and extend to the edges of the cell. During the formation of the network, tropomyosin may function as a cofactor

in the polymerization of the actin filaments that connect the foci, as a length determinant, as a protein that confers structural stability to the newly polymerized filaments, or possibly a combi-

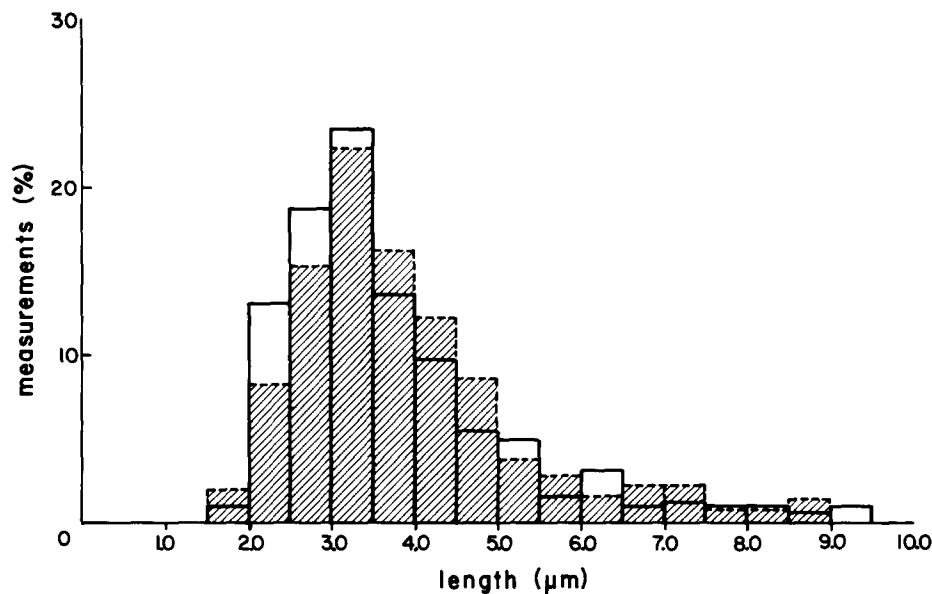


FIGURE 23 A comparison of the distribution of the lengths between the vertices of the networks seen in cells reacted with the actin antibody and photographed with epifluorescence optics (open area) and in live cells photographed with phase contrast optics (shadowed area). Cells having a well-developed network close to the edge of the cell were chosen for measurements. The histogram is derived from 260 measurements made on three different cells reacted with the actin antibody and from 140 measurements made on three different live cells. One of the cells used for the measurements of the lengths between the vertices of the actin network is shown in Fig. 11. One of the live cells used in these measurements is shown in Fig. 22.

nation of these. Tropomyosin is known to be an α -helical protein both in skeletal muscle (5) and in nonmuscle cells (6, 7), and the extended configuration of this molecule may function to induce actin filaments to assemble in straight filamentous bundles. In this way the absence of tropomyosin from the fluorescent foci may allow actin filaments in these foci to have a more flexible configuration and interact with α -actinin in order to form nucleation centers and direct the outward polymerization of the filament bundles that extend from the vertices of the network to the edges of the cell. This latter type of fiber bundles contains all three proteins, actin, α -actinin, and tropomyosin. They are in general much longer than the fibers that connect the vertices of the network (Figs. 8 *a*, 10, and 19), and by both immunofluorescence and morphological criteria they appear indistinguishable from the actin fiber bundles of the fully spread out cells. Thus, they represent the beginning of the formation of these filament bundles. If the vertices of the network act as nucleation centers for the polymerization of these filament bundles towards the edges of the cell, then it is tempting to speculate that the orientation (polarity) of the filaments within a

bundle (as judged by the orientation of the arrowheads formed on actin filaments by heavy meromyosin) would point towards the edge of the cell. In contradistinction to the orientation of these filament bundles, the filaments within the filament bundles that connect the vertices of the network would have opposite (antiparallel) polarities, pointing towards each other away from the vertices, thus resembling the organization of actin filaments within a muscle sarcomere. This possibility becomes even more plausible if one considers that the actin filaments within a sarcomere of a skeletal myofibril are organized with opposite polarities between foci that also contain actin and α -actinin (Z line) (14, 20).

The localization of the vertices of the network are presently undetermined, but the possibility exists that at least some of them may be attached either to the cell membrane or onto any of the membranous organelles within the cell such as the endoplasmic reticulum or the Golgi apparatus. Figs. 12–21 indicate that occasionally the fully developed network covers the whole volume of the spreading cell and may assume the shape of a dome that encompasses the nuclear region.

The network is only transiently formed during cell spreading, but its fate in the fully spread out cell is as yet undetermined. Although the appearance of such a network during the cell cycle is also unknown, it seems reasonable to suppose that such a network may also form both when a cell rounds up for mitosis and when the cell spreads after it has gone through mitosis.

The unknowns in the assembly and organization of these actin filament bundles are still numerous, and α -actinin and tropomyosin may be only two of the many structural or regulatory molecules involved in these processes. In particular, since nonmuscle cells also contain myosin (1, 2), this molecule may also play a specific role in the transient formation of this network as well as the actin fiber bundles of the fully spread out cells, and immunofluorescence studies are currently in progress to determine its role in this process. However, the results reported here demonstrate that α -actinin and tropomyosin are intimately involved in the structural organization of actin-filament bundles in nonmuscle tissue culture cells. At present, we cannot exclude the possibility that the organization of these filament bundles in different nonmuscle cells takes place in several distinct ways. Yet, the study of the network-forming cells allows the investigation of at least one underlying mechanism of fiber bundle formation, which may turn out to be a fundamental and general intermediate in the assembly and organization of actin filament bundles in nonmuscle cells.

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